

A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy

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A mutation equivalent to P102L in the human PrP gene, associated with Gerstmann–Straussler syndrome (GSS), has been introduced into the murine PrP gene by gene targeting. Mice homozygous for this mutation (101LL) showed no spontaneous transmissible spongiform encephalopathy (TSE) disease, but had incubation times dramatically different from wild-type mice following inoculation with different TSE sources. Inoculation with GSS produced disease in 101LL mice in 288 days. Disease was transmitted from these mice to both wild-type (226 days) and 101LL mice (148 days). In contrast, 101LL mice infected with ME7 had prolonged incubation times (338 days) compared with wild-type mice (161 days). The 101L mutation does not, therefore, produce any spontaneous genetic disease in mice but significantly alters the incubation time of TSE infection. Additionally, a rapid TSE transmission was demonstrated despite extremely low levels of disease-associated PrP.

Keywords: gene targeting/prion diseases/PrP mutations/spontaneous prion disease/transmissible spongiform encephalopathies

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases that are also infectious. It has been hypothesized that these diseases are attributable to a conformational change in the prion protein (PrP), which results in a change from a predominantly α -helical protein to a β -sheet form (Prusiner, 1996; Weissmann, 1996). PrP, which is converted from the normal cellular form of the protein (PrP^C) to the protease-resistant, disease-specific form (PrP^{Sc}) during the infectious process (Prusiner, 1991), has been proposed to be the infectious agent (Griffith, 1967). The prion hypothesis predicts that PrP^{Sc} can propagate its own conversion

by acting as a template or a seed (Jarrett and Lansbury, 1993), allowing further conversion of PrP^C to PrP^{Sc} to occur.

Polymorphisms at amino acids 108 and 189 in the murine PrP gene have a major influence on incubation time of scrapie in mice (Moore *et al.*, 1998). Polymorphisms at amino acids 129 and 219 are associated with altered incubation time or susceptibility to human TSEs (Palmer *et al.*, 1991; Goldfarb *et al.*, 1992). Polymorphisms at amino acids 136 and 171 in the PrP gene are associated with susceptibility to TSE disease in sheep (Goldmann *et al.*, 1994). The mechanism by which these mutations lead to altered susceptibility or incubation periods has not been defined, but it has been proposed that the human polymorphisms may be present in a site involved in the conformational transition from PrP^C to PrP^{Sc} (Glockshuber *et al.*, 1999).

In vitro assay systems (Priola *et al.*, 1994) and mouse models (Scott *et al.*, 1992) have suggested that homology between an infectious and endogenous PrP molecule facilitates the efficient conversion of PrP^C to PrP^{Sc}, allowing TSE disease to develop in the host. By introducing an appropriate PrP gene into transgenic mice, the species barrier can be overcome, as demonstrated with transgenic mice expressing a hamster PrP gene, which were shown to be susceptible to hamster strains of scrapie, in contrast to wild-type mice (Scott *et al.*, 1989).

While the above mutations alter the susceptibility of an animal following exposure to an infectious agent, a number of point mutations and insertions in the human PrP gene apparently lead to spontaneous genetic disease (Prusiner, 1997; Parchi *et al.*, 1998), many of which have been transmitted subsequently to rodents and primates (Tateishi and Kitamoto, 1995; Young *et al.*, 1999). It has been suggested that inherited human TSEs result from mutations in the PrP gene leading to amino acid changes that destabilize the three-dimensional structure of the PrP protein (Cohen *et al.*, 1994; Huang *et al.*, 1994; Harrison *et al.*, 1997) and that this inherent instability makes the PrP^C protein more likely to convert to and accumulate as PrP^{Sc}. A transgenic mouse overexpressing a murine PrP gene with a Leu101 mutation was shown to develop disease spontaneously (Hsiao *et al.*, 1990; Telling *et al.*, 1996), suggesting that the 101L mutation may indeed result in an unstable PrP protein. In addition, the resultant spontaneous disease was transmitted to transgenic mice expressing the same transgene but not to wild-type mice (Hsiao *et al.*, 1994), suggesting a requirement for homology between PrP molecules for transmission of the disease to occur.

PrP has also been implicated in the pathological process leading to neurodegeneration. PrP^{Sc} accumulates in the brain during disease, but grafting tissue from wild-type mice into the brain of PrP null mice has shown that

accumulation of PrP^{Sc} is not sufficient to lead to the development of pathology in the brain in the absence of PrP^C (Brandner *et al.*, 1996). PrP^{Sc} accumulation has not been detected in all TSE disease. Although PrP accumulates in the brains of the 101 transgenic mice described above, it does not have the characteristic protease resistance associated with PrP^{Sc} (Hsiao *et al.*, 1990; Telling *et al.*, 1996). Primary transmission of bovine spongiform encephalopathy (BSE) agent to mice has been described in the apparent absence of PrP^{Sc} accumulation (Lasmezas *et al.*, 1997). Spontaneous neurodegenerative disease has also been reported in transgenic mice with an AV3 substitution in PrP in which an abnormal transmembrane form of PrP protein accumulates rather than PrP^{Sc} (Hedge *et al.*, 1998), although transmissibility of disease from these mice has not been reported.

Although certain mutations in PrP have been shown to alter the incubation period of TSE disease (Moore *et al.*, 1998), the mechanism by which this effect is achieved has not been defined. Here we describe the introduction of a 101L mutation into the endogenous murine PrP gene. This mutation does not lead to the development of spontaneous TSE disease in these mice, but dramatically alters the incubation times of disease following exposure of the 101LL mice to different strains of agent. One of the resulting TSE diseases can be transmitted to both wild-type and mutant mice with short incubation periods, despite extremely low amounts of PrP^{Sc} in the inoculum.

Results

Gene expression from the *Prnp*^{a101} allele

A two-step double replacement gene targeting strategy was used to alter *Prnp*^a exon 3 in HM-1, a 129/Ola murine embryonic stem cell line. Gene targeting was used to alter specifically amino acid 101 (equivalent to amino acid 102 in human PrP) from the wild-type proline (101P) residue to a leucine (101L), to generate *in situ* a modified *Prnp*^a allele expressing the mutant PrP gene (Moore *et al.*, 1995). The targeted allele has been designated *Prnp*^{tm1Edin} referred to in this text by the more descriptive name *Prnp*^{a101L} (Figure 1A). HM-1 embryonic stem (ES) clones carrying a *Prnp*^{a101L} mutant allele were used to produce chimeric mice (Moore *et al.*, 1995). A chimera, which transmitted the mutant allele, was used to generate progeny heterozygous for the *Prnp*^{a101L} allele (101PL), which were then inter-bred to produce progeny homozygous for the *Prnp*^{a101L} allele (101LL). Wild-type mice are referred to as 101PP.

The structure of the targeted *Prnp*^{a101L} allele in the mice was investigated by Southern blot analysis (Figure 1B) using probes derived from intron 2 and exon 3 (Figure 1A). The 101L mutation changes CCC-Pro to CTC-Leu, creating an additional *DdeI* site within exon 3. No other alteration was detected in the targeted allele. The entire *Prnp* coding region from the 101LL mice was sequenced to confirm that no mutations, other than the desired one, had been introduced during the construction of the mice (data not shown). These studies confirm that the *Prnp*^{a101L} allele has undergone no detectable deletions, insertions or rearrangements during the gene targeting process or during the production of the mice. The 101PL mice were crossed with CB20 mice to produce an outbred

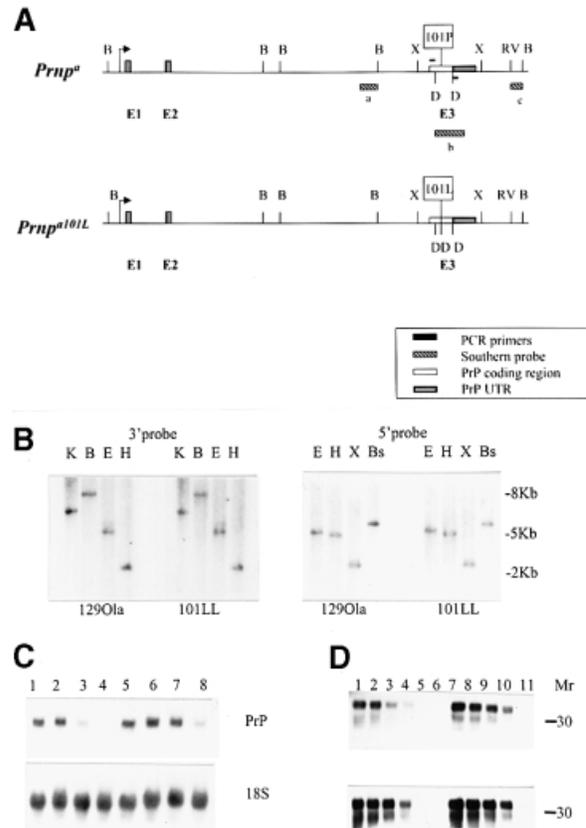


Fig. 1. (A) Gene structure and gene expression from the *Prnp*^{a101L} allele. Restriction map of the wild-type 129/Ola *Prnp*^a allele and the targeted *Prnp*^{a101L} alleles. Probes are shown as hatched boxes. The *Prnp*^a allele yields 151 and 613 bp *DdeI* exon 3 fragments and the *Prnp*^{a101L} allele yields 464, 151 and 149 bp *DdeI* fragments from exon 3 due to the additional *DdeI* site created by the mutation. The following *Prnp* probes were used: probe a, a 884 bp PCR product of intron 2 from 25 004 to 25 887 bp (DDBJ/EMBL/GenBank accession No. U29186); probe b, a 936 bp *KpnI*-*EcoRI* fragment from exon 3; and probe c, a 700 bp *EcoRV*-*BamHI* fragment at the 3' end of the *Prnp* gene. Abbreviations: *BamHI* (B), *XbaI* (X), *DdeI* (D), *EcoRV* (RV), E1, exon 1; E2, exon 2; E3, exon 3. (B) Genomic Southern analysis of the *Prnp*^{a101L} allele. Genomic DNA was isolated from 101LL and 101PP mice and digested with *KpnI* (K), *BamHI* (B), *EcoRI* (E), *HindIII* (H), *XbaI* (X) and *BstEII* (Bs). Following digestion, the fragments were separated on a 1% agarose gel blotted on to Genescreen plus (Dupont) and hybridized with probe c (3' end of the *Prnp* gene) and probe a (5' end of the *Prnp* gene). These data, together with exon 3 nucleic acid sequence (data not shown), confirm that the targeted *Prnp*^{a101L} allele is indistinguishable from the parental 129/Ola *Prnp*^a allele, with the exception of the engineered PrP codon 101L alteration. (C) Northern analysis of PrP mRNA in the brain. Total brain RNA (20 µg) probed with a 936 bp *KpnI*-*EcoRI* mouse PrP exon 3 DNA probe. Lanes 1 and 2, wild-type 129/Ola (101PP); lane 3, heterozygous PrP null (101P⁻) to demonstrate that a 50% reduction in mRNA was detectable; lane 4, PrP⁻; lanes 5-7, mice with two copies of the targeted *Prnp*^{a101L} allele (101LL); lane 8, mice with one copy of the *Prnp*^{a101L} allele and a null allele (101L⁻). Mouse 18S rRNA reprobe of the membrane. Quantitation of the 18S rRNA signal was determined using PhosphorImager technology (Molecular Dynamics), and these values were used to correct for loading variations. (D) Western blots of crude brain homogenates from mice expressing wild-type *Prnp*^a and gene-targeted *Prnp*^{a101L} alleles. Samples were resolved on SDS-PAGE and transferred to PVDF membrane. PrP was detected with a rabbit anti-mouse PrP polyclonal serum 1A8 (top) or mouse monoclonal 8H4 (bottom) and visualized with either HRP-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibodies and a chemiluminescence detection kit. Lanes 1-5, mouse with two copies of the *Prnp*^{a101L} allele (101LL); lanes 7-11, wild-type mouse (101PP). Lanes 1 and 7, 100 µg; lanes 2 and 8, 75 µg; lanes 3 and 9, 50 µg; lanes 4 and 10, 25 µg; and lanes 5 and 11, 1 µg wet weight tissue equivalents.

line of mice with the mutant allele as well as being maintained on an inbred 129/Ola background. All TSE inoculation experiments were carried out using inbred mice.

The level of expression of the PrP gene from the targeted allele (*Prnp^{a101L}*) was assessed and compared with the wild-type gene. Northern blot analysis detected similar levels of PrP mRNA in mice with the mutant *Prnp^{a101L}* allele (101LL) and in wild-type mice (101PP) (Figure 1C). Western blot analysis using both monoclonal and polyclonal PrP antibodies, however, has indicated that the steady-state level of the PrP protein in 101LL mice is apparently lower than that in wild-type mice (Figure 1D). Accurate quantification of the difference in amount of PrP between the two lines of mice has proved difficult by Western blot analysis, but a more quantitative assay system is being developed to address this question. This reduction in PrP in 101LL mice may be a result of altered processing or stability of the mutant protein. Alternatively, altered conformation may lead to differences in the ability of the antibodies to bind to the mutant PrP protein and thus lead to an apparent reduction in the protein levels detected. The different PrP protein levels in the 101LL mice most probably result directly from the 101L mutation, which has been introduced into the murine gene, since sequencing and Southern blot analysis of the PrP gene did not detect any differences between the 101LL and 101PP mice except for the 101L mutation. Additional evidence that suggests that the alteration in PrP protein level may be a specific effect of the 101L mutation is provided by the previous gene targeting experiments in which we introduced alterations into amino acids L108F and T189V. Mice with these alterations were shown to have levels of PrP mRNA and protein identical to the wild-type mice (Moore *et al.*, 1998). Why this mutation should lead to an altered level of PrP is currently under investigation, but for the purposes of assessing TSE incubation times in the 101LL and 101PL mice reported here, it is important to allow for the apparent reduction in level of PrP protein in the 101LL mice. This is because it has been shown that mice with only one copy of the wild-type PrP gene, and thus with reduced levels of PrP protein, have longer incubation times than wild-type mice following inoculation with different strains of TSE (Manson *et al.*, 1994).

The *Prnp^{a101L}* allele is not sufficient for spontaneous neurodegenerative disease

Both inbred and outbred mice (>200 animals) carrying one or two copies of the *Prnp^{a101L}* allele showed no clinical signs of TSE disease up to 899 days of age (63 of which were >700 days old). The brains of all culled mice have been examined for pathological signs of subclinical TSE. Animals over 350 days showed some vacuolation limited to white matter regions of the brain, consistent with ageing vacuolation and typical of control groups.

In order to assess whether there had been any accumulation of PrP in the brain, despite the absence of clinical signs of disease, brains from 27 mice both homozygous and heterozygous for the *Prnp^{a101L}* allele (631–888 days old) were examined by immunocytochemical and Western blot analysis. No PrP deposition was detected in the brains by immunocytochemical analysis (not shown) and no

PrP^{Sc} was detected by Western blot analysis after long exposure times of immunoblot to X-ray film (Figure 6A).

Brains from mice homozygous for the *Prnp^{a101L}* allele were used as an inoculum to assess whether, despite the absence of clinical or pathological signs of TSE, they carried spontaneously and endogenously generated TSE infectivity. A pool of three brains from mice (101LL) over 700 days old was inoculated intracerebrally into homozygous (101LL), heterozygous (101PL) and wild-type 129Ola (101PP) mice. At the time of writing, these mice have shown no clinical signs of TSE disease (600 days after inoculation).

The 101L mutation alters the incubation time of TSE disease

Inbred 129/Ola mice carrying no (101PP), one (101PL) or two (101LL) copies of the *Prnp^{a101L}* allele were inoculated with brain homogenate from a patient who died of Gerstmann–Straussler syndrome (GSS) (Table I). The entire coding region of the PrP gene of this patient was sequenced and was shown to be heterozygous for the 102L mutation, homozygous for 129M and carried no other mutation in the PrP gene. Only one of eight wild-type mice showed clinical signs of disease 456 days after inoculation. Vacuolation was apparent in the cerebellar and midbrain white matter regions in the brain of this mouse, but no significant grey matter vacuolation was detected. The remaining seven wild-type mice showed no signs of clinical disease or significant vacuolar pathology in either white or grey matter regions of the brain up to 701 days post-infection. The brains of these mice were also devoid of PrP by immunocytochemical analysis and no PrP^{Sc} was detected by Western blot analysis (Figure 6B).

Mice carrying two copies of the *Prnp^{a101L}* allele (101LL) all developed clinical signs of disease and were culled between 254 and 317 days after infection. The clinical phase extended over 4–6 weeks and included ataxia, hind limb paralysis and marked kyphosis. Vacuolar pathology of both grey and white matter was evident in the brains of these mice (Figure 2A). After extensive immunocytochemical analysis of sections at different levels throughout the brain, using two different polyclonal antibodies (1B3 and 1A8), abnormal accumulation of PrP could not be detected in five of the brains (Figure 3B, Table II). This was despite the fact that all cases had marked vacuolation in the thalamus, septum and hypothalamus and severe white matter vacuolation of the midbrain and cerebellar regions. Minor diffuse deposits of PrP were detected in five other cases, restricted to the thalamus and inner cortical layer. Two of these cases also showed aggregates of PrP in the corpus callosum near the site of injection, perhaps due to retention of inoculum in this region.

Although no PrP deposition was detected immunohistochemically in five of the 101LL mice, the analysis may not have been sensitive enough to detect very low levels of PrP in the brain. The histochemical processes required here for the treatment of the tissues (e.g. formic acid treatment) have been reported to lead to a loss of PrP^C from some tissues (Kitamoto *et al.*, 1991) and may also lead to some loss of PrP^{Sc}. Western blot analysis of the brains of the 101LL mice infected with GSS readily detected PrP^C. Polyclonal antisera did not detect any protease-resistant PrP in these brains on Western blots

Table I. Incubation time of disease following inoculation with GSS, GSSLL and ME7

Inoculum	Mouse strain	No. with clinical TSE	No. without clinical TSE	Incubation time ± SEM
GSS (10 ⁻¹)	101PP	0	1	
	101PL	4	0	479 ± 29
	101LL	2	0	254 ± 0
GSS (10 ⁻²)	101PP	1	6	456
	101PL	10	8	450 ± 12
	101LL	15	0	288 ± 4
GSSLL (10 ⁻²)	101PP	16	0	226 ± 3
	101PL	20	0	201 ± 3
	101LL	18	0	148 ± 2
ME7 (10 ⁻²)	101PP	10	0	161 ± 2
	101PL	29	0	353 ± 4
	101LL	18	0	338 ± 8

Mice with no (101PP), one (101PL) and two (101LL) copies of the *Prnp*^{a101L} allele were inoculated with primary GSS (GSS), GSS passed through a 101LL mouse (GSSLL) and ME7. The inoculum was injected intracerebrally at 10⁻¹ or 10⁻² dilution.

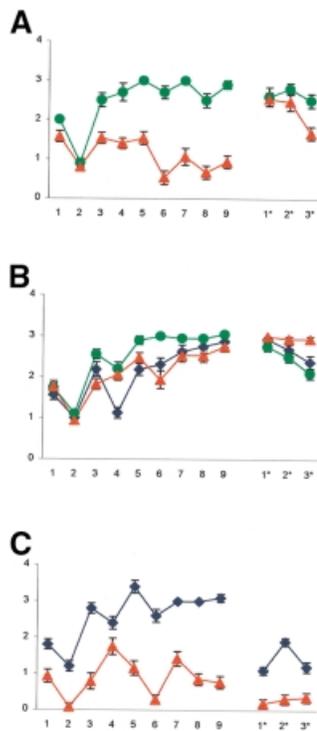


Fig. 2. Mice with one and two copies of the *Prnp*^{a101L} allele and wild-type mice were inoculated with brain homogenate from: (A) a GSS patient with the 102L mutation; (B) a 101LL mouse terminally infected with GSS; (C) ME7. The extent of vacuolar change in the brain was assessed semi-quantitatively in nine areas of grey matter and three of white matter by lesion profiling as described (Fraser and Dickinson, 1967, 1968). Circles (green) are lesion profiles of mice at the terminal stages of disease with one copy of the gene-targeted allele *Prnp*^{a101L} (101PL), triangles (red) are mice with two copies of the *Prnp*^{a101L} allele (101LL) and diamonds (blue) are wild-type (101PP) mice. Lesion profiles were constructed (using a minimum of 10 animals in each group) on a scale of 0–5, and mean scores for each area are shown graphically (error bars ± SEM). Note: lesion scores without error bars represent scoring areas where there was no variation in lesion scores. Lesion profile scoring areas: grey matter areas: 1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, medial thalamus; 6, hippocampus; 7, septum; 8, cerebral cortex; 9, forebrain cerebral cortex. White matter areas: 1*, cerebellar white matter, 2*, midbrain white matter 3*, cerebral peduncle.

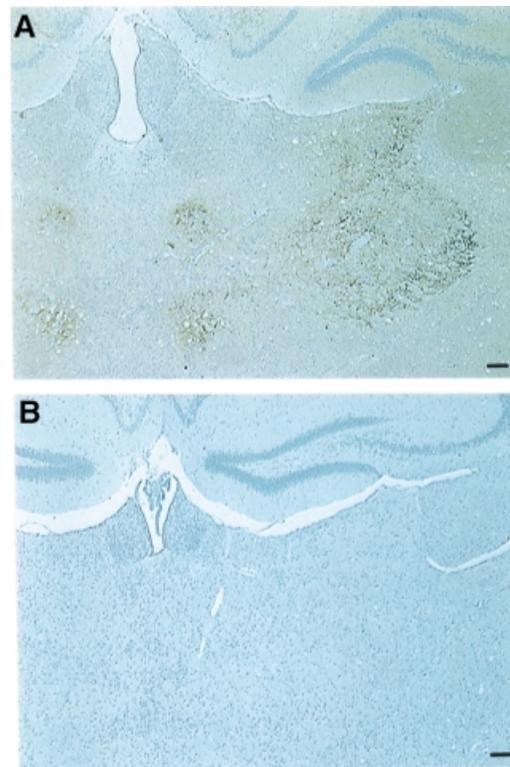


Fig. 3. Mice with one and two copies of the *Prnp*^{a101L} allele and wild-type mice were inoculated with brain homogenate from a GSS patient with the 102L mutation. The brains of the animals were examined either at the terminal stage of the disease for animals which developed TSE disease or at the end of their lifespan for animals with no clinical signs of disease. PrP deposition in the hippocampus and thalamus detected by immunocytochemical analysis using two polyclonal antibodies 1A8 (not shown) and 1B3 (Farquhar *et al.*, 1989). (A) PrP was detected in the thalamus of 101PL mice inoculated with GSS in both clinically positive and negative (as shown here at 639 days) animals, but (B) was barely detectable at the terminal stage of disease (254 days) in 101LL mice inoculated with GSS. Bar, 100 µm.

(data not shown). However, use of a monoclonal antibody (8H4) and prolonged exposure times of the immunoblot to X-ray film did detect very low levels of protease-resistant PrP in the 101LL GSS-infected mice (Figure 6B). This protease-resistant PrP was not present in uninfected or aged 101LL mice after similarly long exposure times and was also not detected in PrP null mice (Figure 6B).

Table II. PrP deposition detected by immunocytochemistry in GSS primary transmission

Mouse genotype	No.	Clinical TSE	Vacuolar pathology	PrP deposition
101PP	7	-	-	-
101PP	1	+	-	-
101PL	1	-	-	-
101PL	1	-	-	+
101PL	3	-	+	+
101PL	6	+	+	+
101LL	5	+	+	-
101LL	5	+	+	+

Mice with no (101PP), one (101PL) and two (101LL) copies of the *Prnp^{a101L}* allele inoculated with infectious material from a patient with GSS were examined for PrP accumulation by immunocytochemistry using two polyclonal antibodies (1B3 and 1A8). The amount of PrP deposited in the positive 101PL cases was variable. Only very low levels of PrP were detected in the positive 101LL cases.

It is therefore likely to represent extremely low levels of PrP^{Sc} in the infected brains. Accumulation of disease-specific PrP does not appear to be a significant feature of the TSE pathology in this experimental model.

Mice carrying one copy of the wild-type and one copy of the mutant allele (101PL) varied considerably in terms of clinical and pathological TSE (Tables I and II). One group showed no clinical signs of TSE disease (death between 537 and 701 days from other causes) and had no significant vacuolar pathology; a second group showed no clinical signs of disease (death between 537 and 646 days from other causes) but had significant grey and white matter vacuolation. A third group developed TSE disease between 411 and 540 days and had severe vacuolation in grey and white matter regions (Figure 2A). Immunocytochemical analysis of clinically negative 101PL mice revealed one animal with no detectable PrP and one with PrP deposition. All clinically positive mice showed PrP immunostaining. The amount of PrP deposited in the 101PL mice varied from minor cortical PrP to severe deposition throughout the thalamus and cortex and did not appear to relate to the clinical status of the animals (Figure 3A, Table II). PrP^{Sc} was detected by Western blot analysis in all clinically positive 101PL mice inoculated with GSS but not in the two clinically negative animals examined (Figure 6B).

In mice with only one copy of the mutant gene (101PL), the incubation period of disease is less constant than in the homozygous mice (101LL). Prolonged and variable incubation periods in the heterozygous mice may reflect a copy number effect, with a single copy of the mutant gene leading to longer incubation times. The presence of the wild-type gene in these mice may be interfering with the disease process associated with the mutant allele. The mutant allele (*Prnp^{a101L}*) has now been crossed on to a PrP null background so that the effect of a single copy of the mutant gene can be assessed in the absence of the wild-type gene.

Disease can be transmitted from a GSS-infected 101LL mouse to mice with or without the *Prnp^{a101L}* allele

A brain homogenate was prepared from a 101LL mouse infected with GSS that had been culled at the terminal stage

of disease (254 days) with no apparent PrP deposition. The brain used to prepare the inoculum is that shown in Figure 3B. The inoculum (GSSLL) was injected intracerebrally into mice of the three genotypes (101PP, 101PL and 101LL). Disease was transmitted to all three groups of animals, with homozygous 101LL mice having the shortest incubation period. Significantly, even the wild-type (101PP) mice developed TSE disease within 226 days (Table I). Thus despite the failure to detect any PrP accumulation by immunohistochemical analysis and the extremely low levels of protease-resistant PrP detected by Western blot analysis in the brains of 101LL mice infected with GSS, the GSSLL inoculum prepared from it was able to transmit TSE disease rapidly to mice both with and without the 101L mutation.

Similar patterns and levels of vacuolation were detected in the brains of mice of all three genotypes, with particularly high levels being present in white matter areas such as the cerebellum and cerebral peduncle (Figures 2B and 4A). The heterozygous animals tended to have slightly higher scores in a number of regions and the wild-type mice had considerably less vacuolation in the hypothalamus (region 4) than the other groups (Figure 2B).

In contrast to the uniformity of vacuolation, the deposition of PrP in the three genotypes of mice showed marked differences (Figure 4B). The most striking areas of PrP deposition were the thalamic dorsal lateral geniculate nuclei and the corpus callosum and subcallosal regions. The wild-type mice showed marked PrP immunoreactivity in the thalamus and in the corpus callosum. The heterozygous animals showed variable PrP immunoreactivity patterns in the thalamus, ranging from low to moderate levels of PrP deposition. Grainy PrP deposits were present in the stratum radiatum coupled with diffuse PrP immunoreactivity throughout the entire hippocampus.

In contrast to the primary GSS infection of 101LL mice, PrP accumulation was evident in most of the 101LL mice inoculated with GSSLL (Figure 4B). All but one case showed deposition of PrP throughout the thalamic region. Diffuse deposits of PrP were also detected in the stratum radiatum and four cases also showed diffuse PrP coupled with clusters of aggregates throughout the corpus callosum. There was marked variation in the amount of PrP detected; one case showed only minor diffuse PrP immunoreactivity limited to a small focal area of the thalamus, while another with an incubation period of only 1 day more had extensive PrP accumulation. PrP^{Sc} was detected by Western blot analysis in extracts from all three groups of animals, although it appeared to vary in quantity, with less being detected in the 101LL mice than in the other two groups (101PP and 101PL) (Figure 6C).

101LL mice have long incubation times with ME7

Inoculation with a murine strain of scrapie, ME7, produced long incubation periods in mice with one (101PL) and two (101LL) copies of the *Prnp^{a101L}* gene compared with the wild-type mice (101PP) (Table I). Although there was overlap between the incubation periods of heterozygous (101PL) and homozygous (101LL) mutant mice, the incubation periods in heterozygous mice tended to be longer than in homozygous mice. This is similar to the effects seen in the F₁ progeny of crosses between strains of wild-type mice differing in their *Prnp* alleles when

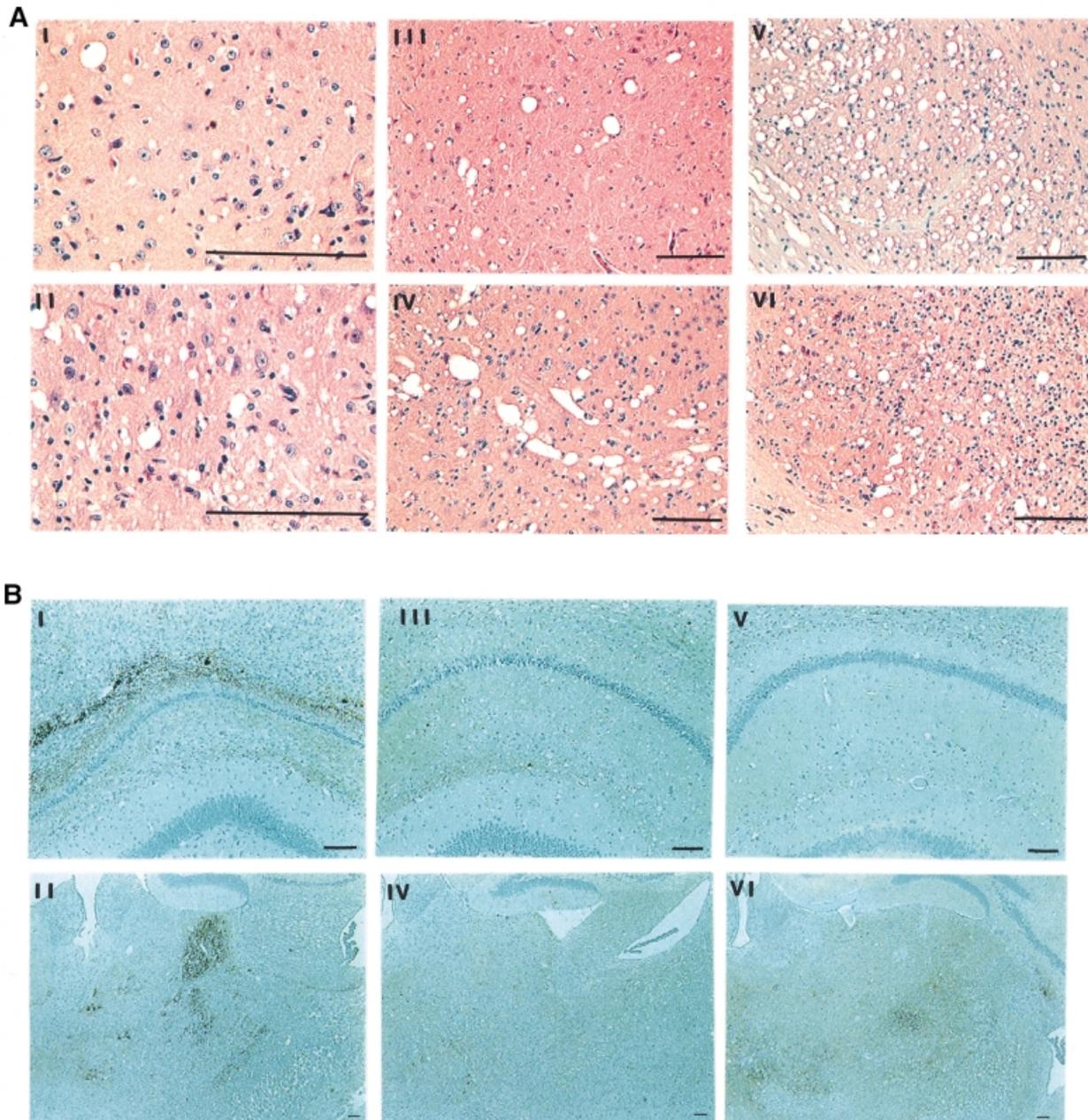


Fig. 4. Disease can be transmitted from GSS-affected 101LL mice with no detectable PrP accumulation. Mice with one and two copies of the *Prnp*^{101L} allele and wild-type mice were inoculated with brain homogenate from 101LL mice infected with GSS (the brain used for inoculation is that shown in Figure 3B, in which PrP accumulation was not detectable at the terminal stage of disease). The brains of the animals of each genotype were examined at the terminal stage of the disease. **(A)** Vacuolar changes were detected in both grey and white matter regions of the brain. The heterozygous mice (101PL) had more vacuolation than the other two groups in a number of areas including the thalamus: (i) 101LL and (ii) 101PL. Vacuolation in white matter was detected in all three genotypes but was most severe in the 101LL mice in the midbrain white matter, (iii) 101LL and (iv) 101PP, and in the cerebral peduncle, (v) 101LL and (vi) 101PP. Bar, 100 μ m. **(B)** PrP detected by immunocytochemistry in the brains of the three genotypes of mice at the terminal stages of disease using polyclonal antibodies 1A8 (not shown) and 1B3. (i) The hippocampal region in 101PP mouse showed extensive neuronal loss of the CA1 pyramidal layer and PrP deposition in the corpus collosum. (iii) Minor CA1 pyramidal neuronal loss and minimal deposits of PrP in the corpus collosum were detected in the heterozygous (101PL) mice whereas in the 101LL mice (v) there was no evidence of CA1 neuronal loss and only minimal PrP accumulation was detected. (ii) Extensive PrP accumulation was detected in restricted areas of the thalamus in 101PP mice. (iv) Lower levels were detected throughout the thalamic region of the 101PL mice, and (vi) in the 101LL mice extensive PrP deposition was detected throughout the thalamus. Bar, 100 μ m.

inoculated with a number of different strains of scrapie (Bruce *et al.*, 1991) although not when inoculated with ME7. The prolonged incubation periods in the mutant mice may be due in part to the reduced level of PrP, but this is unlikely to account for such long incubation periods (Manson *et al.*, 1994) and the mutation must also be having a direct effect on the length of incubation time.

The ME7-infected wild-type (101PP) mice had diffuse PrP deposits throughout the brain (Figure 5) and vacuolation was also observed in all regions of the brain (Figure 2C). Marked neuronal loss was observed in the CA1 region of the hippocampus. In contrast, the neuropathology in the 101LL mice infected with ME7 was more restricted in terms of both vacuolation

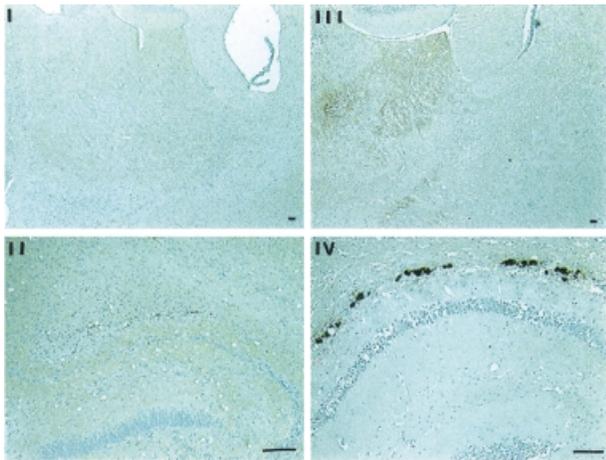


Fig. 5. 101LL mice have long incubation times with ME7. Mice with one and two copies of the *Prnp*^{101L} allele and wild-type mice were inoculated with ME7 passaged in *Sinc s7* mice. The brains of animals of each genotype were examined at the terminal stage of the disease. PrP detected by immunocytochemistry in the brains of the mice at the terminal stages of disease using polyclonal antibodies 1A8 (not shown) and 1B3. Diffuse deposits of PrP were detected throughout the brain in the 101PP mice (i) in the thalamus (101PP) and (ii) the hippocampus and corpus callosum (101PP), whereas in the 101LL mice PrP deposition showed a more restricted pattern (iii) with extensive accumulation in some areas of the thalamus (101LL) and (iv) a large number of PrP aggregates along the entire length of the corpus callosum (101LL). Bar, 100 μ m.

(Figure 2C) and PrP deposition (Figure 5). In 101LL mice, diffuse extracellular PrP was most notable in the thalamus and hypothalamus. A large number of PrP aggregates were seen along the entire length of the corpus callosum and diffuse aggregates were observed in all cortical layers. Neuronal loss was evident in the hippocampal CA1 pyramidal cell layer but not to the same extent as that observed in the wild-type mice. PrP^{Sc} was detected by Western blot analysis in all three genotypes with wild-type mice (101PP) showing quantitatively more PrP^{Sc} than mice carrying one or two copies of the *Prnp*^{101L} allele (101PL and 101LL) (Figure 6D).

The different disease profiles in the 101PP and 101LL mice may result from different PrP alleles targeting pathology to different brain regions, although such extreme differences in pathology have not been observed previously between wild-type PrP alleles. Alternatively, these mice may have selected a variant strain of ME7, as has been observed previously for another scrapie strain after changing the genotype of the mouse in which it was passaged (Bruce and Dickinson, 1987). These possibilities are currently under investigation.

Discussion

On the basis of a number of family studies, GSS, linked to the 102L mutation in human PrP, has been described as a genetic disease with an autosomal dominant mode of inheritance and high penetrance (Hsiao *et al.*, 1989; Speer *et al.*, 1991). Introduction of this mutation *in situ* into one or both of the endogenous murine PrP genes has not resulted in an inheritable spontaneous TSE in mice. Perhaps differences in other amino acids between the mouse and human PrPs block the specific effects of the human 102L appearing

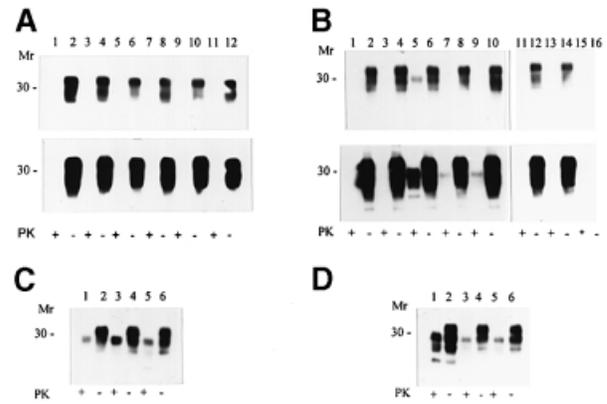


Fig. 6. Western analysis of brain homogenates for PrP^{Sc} using 8H4 monoclonal antibody. (A) Brains of uninoculated mice: lanes 1 and 2, 101PL (794 days old); lanes 3 and 4, 101PL (806 days old); lanes 5 and 6, 101PL (807 days old); lanes 7 and 8, 101LL (806 days old); lanes 9 and 10, 101LL (815 days old); lanes 11 and 12, 101LL (812 days old). Lanes 1, 3, 5, 7, 9 and 11 are treated with 20 μ g/ml proteinase K; lanes 2, 4, 6, 8, 10 and 12 are samples without proteinase K. Exposure times of 10 s (top) and 10 min (bottom) are shown to demonstrate the absence of PrP^{Sc} in these samples. (B) Mice of each genotype inoculated with brain homogenate from a GSS patient with the 102L mutation. Lanes 1 and 2, 101PP with no clinical TSE; lanes 3 and 4, 101PL with no clinical TSE; lanes 5 and 6, 101PL with clinical TSE; lanes 7 and 8, 101LL at the terminal stage of disease; lanes 9 and 10, 101LL at the terminal stage of disease; lanes 11 and 12, uninfected 101PP; lanes 13 and 14, uninfected 101LL; lanes 15 and 16, uninfected PrP null mice. Lanes 1, 3, 5, 7, 9, 11, 13 and 15 are samples digested with 20 μ g/ml proteinase K; lanes 2, 4, 6, 8, 10, 12, 14 and 16 are samples without proteinase K. Two different exposure times of the Western blot are shown: 10 s (top) and 10 min (bottom). (C) Mice of each genotype inoculated with brain homogenate from a mouse primarily infected with human GSS at the terminal stage of disease (GSSLL). Lanes 1 and 2, 101LL; lanes 3 and 4, 101PL; lanes 5 and 6, 101PP. Lanes 1, 3 and 5 are digested with 20 μ g/ml proteinase K; lanes 2, 4 and 6 are without proteinase K. (D) Mice of each genotype infected with ME7. Lanes 1 and 2, 101PP; lanes 3 and 4, 101PL; lanes 5 and 6, 101LL. Lanes 1, 3 and 5 are digested with 20 μ g/ml proteinase K; lanes 2, 4 and 6 are without proteinase K.

as a disease in mice. On the other hand, in previous studies, overexpression of PrP from multiple copies of a murine–hamster chimeric gene carrying the 101L mutation led to a spontaneous neurodegenerative disease in mice (Hsiao *et al.*, 1990; Hsiao *et al.*, 1994; Telling *et al.*, 1996). It may be that the lifetime of a mouse carrying only one or two copies of the mutant gene is not long enough to enable the stochastic event that results in TSE disease to occur. If this is the case, it would be argued that the familial TSE diseases of humans could only be modelled in mice by overexpression of mutant PrP genes. This possibility cannot be ruled out; however, wild-type levels of expression of a mutant PrP gene recently have been reported to lead to a spontaneous neurological disease in mice (Chiesa *et al.*, 1998), suggesting that high overall levels of expression of PrP are not an absolute requirement for the development of spontaneous disease. Multiple factors may be involved in triggering the disease process, some of which may be determined by local concentrations of PrP in different cell compartments and these may differ as a result of variations in the processing of PrP expressed from different *Prnp* alleles. Whether disease resulting from general or local overexpression of PrP is triggered by the same mechanism in mice as normal levels of expression, remains to be established. That different processes may be operating is suggested by the observation

that disease in the high expressing 101L mice could only be transmitted to mice that also carried the 101L transgene (Hsiao *et al.*, 1994), whereas the infectivity produced in gene-targeted 101LL mice inoculated with GSS, described here, was readily transmissible to wild-type mice in addition to those with *Prnp*^{101L} alleles.

The prion hypothesis, supported by PrP structural analysis, predicts that certain mutations in PrP may result in unstable PrP molecules that are more likely to convert from a normal cellular PrP^C to the abnormal PrP^{Sc}, thus initiating the disease process (Cohen *et al.*, 1994; Huang *et al.*, 1994; Prusiner, 1997). The 102L mutation, in common with a number of other mutations associated with human disease, resides in the apparently unstructured N-terminal region of the PrP protein. The structural importance of this region of PrP may relate to its interaction with either other parts of the PrP protein or other molecules. It has been suggested that altered processing of mutant PrP molecules or alterations in biochemical properties following synthesis may render the protein infectious (Harris and Lehmann, 1996). *In vitro* studies using a recombinant PrP with the 101L mutation did not detect alterations in stability properties (Swietnicki *et al.*, 1998), but alterations in secondary structure have been observed when a recombinant 101L PrP protein was compared with a recombinant wild-type PrP protein (Cappai *et al.*, 1999). While introduction of this mutation into the murine gene described here has apparently led to a reduction in PrP protein levels in the 101LL mice, which might be consistent with altered processing or stability of the mutant protein, these studies have not provided any biological evidence for gross 'instability' of the PrP protein. Indeed when the mutant mice were challenged with the ME7 strain of scrapie, the incubation time to clinical disease was significantly longer than in the wild-type mice and could not be accounted for simply by reduced levels of PrP^C. This suggests that the 101L PrP protein replicates ME7 infectivity more slowly than the wild-type PrP.

The introduction of the 101L mutation into the murine PrP gene has dramatically altered the incubation period of TSEs in the mice that carry the mutant gene. Challenge with an inoculum from a GSS102L patient has led to disease developing in all the mice carrying two copies of this mutation in only 288 days. The presence of the 101L mutation in the murine gene is sufficient to render animals highly susceptible to disease when challenged with brain homogenate from a GSS case. Transmission of infectivity from one species to another usually results in disease in only a low percentage of animals and with a wide range of incubation times on primary passage. The difficulty of transmitting TSE disease between two species is known as the 'species barrier' (Dickinson, 1976). This barrier was observed when wild-type 101PP mice were inoculated with brain material from a GSS patient, with only one out of eight animals developing clinical signs of TSE. In contrast, all the 101LL mice succumbed to disease with similar incubation periods when inoculated with GSS, indicating that the introduction of a single point mutation into the endogenous murine PrP gene has removed a major part of the human to mouse species barrier with GSS infectivity. The remarkably short incubation periods for GSS in the 101LL mice have not been observed when

these mice have been inoculated with other sources of human TSE (experiments in progress).

The results presented here suggest that rather than producing a spontaneous transmissible disease, the substitution of proline by leucine at amino acid 101 in the murine PrP gene renders the animals that carry it more susceptible to human GSS while increasing incubation time with ME7. This altered response is comparable to that observed following switching of codons at 108 and 189 in the murine gene (Moore *et al.*, 1998). Our findings are therefore consistent with the idea that humans carrying 102L may have increased susceptibility to specific strains of TSE infection (e.g. GSS), rather than succumbing to a spontaneous disease, i.e. an apparent genetic disease may be caused by a high susceptibility to a ubiquitous agent.

The identification of PrP^{Sc} in the brain and other tissues is often taken as a definitive marker of TSE disease. In many TSEs, a close correlation exists between the level of infectivity and the level of PrP^{Sc}. There are, however, a number of examples in which the particular characteristics of disease are not associated with high levels of PrP^{Sc} (Hsiao *et al.*, 1994; Lasmezas *et al.*, 1997; Hedge *et al.*, 1998). Nevertheless, the apparent absence of PrP^{Sc} has been equated with a low titre or absence of infectivity. Several animals carrying the 101L mutation and showing clinical signs following infection with human GSS inoculum had extremely low levels of PrP. The deposition of PrP in the brains of these animals was barely detectable despite extensive immunocytochemical analysis and was only detected by Western blot analysis following long exposure times. Brain material from one of the mice succumbing to disease after challenge with the human GSS source was inoculated into both transgenic (101PL and 101LL) and non-transgenic (101PP) mice, and disease developed rapidly in all recipients. The low level of PrP^{Sc} detected by Western blot analysis in the brain of a 101LL mouse on first passage with GSS (Figure 6B) would correspond to a very low titre of infectivity in other well-characterized murine models of scrapie and would be associated with long incubation times on subsequent passage in mice. Without a full titration (which is now in progress), we are unable to show formally that there is a high titre of infectivity in the first passage brain, but the short incubation periods in mice receiving this inoculum would suggest that a high titre of infectivity was present in this brain with very low levels of PrP^{Sc}. Apparently, the lack of detectable PrP^{Sc} in a tissue may not always represent a reliable marker for the absence of TSE infection.

Materials and methods

Generation of the mice heterozygous and homozygous for the *Prnp*^{101L} allele

PrP codon 101 alteration was introduced into HM-1 ES cells as described (Moore *et al.*, 1995). Briefly, the gene-targeting vector was constructed using isogenic 129/Ola *Prnp*^a exon 3-flanking sequence derived from an HM-1 genomic library in λ DASH II (Stratagene). PrP codon 101 alteration was introduced into a 934 bp *KpnI*-*EcoRI* exon 3 fragment containing the open reading frame (ORF) by the Kunkel method (Kunkel *et al.*, 1987) and ligated in a single step with 5' and 3' homologous sequence derived from p129PrP, a 8.5 kb *BamHI* genomic clone spanning 129/Ola exon 3. The ORF encoding PrP in the targeting vector was sequenced immediately prior to use and this confirmed the presence of the desired *Prnp* alterations and the absence of cloning artefacts or other

mutations. 129/Ola HM-1 ES cells with the targeted *Prnp*^{101L} allele were introduced into BALB/c 3.5 day blastocysts and transferred to pseudopregnant MF1 recipients as described (Thompson *et al.*, 1989). Chimeras were mated with inbred 129/Ola (129/OlaHsd, Harlan, UK) and germline pups were investigated for the targeted allele by PCR. 129/Ola littermates heterozygous for the targeted allele were crossed to generate mice homozygous for the *Prnp*^{101L} allele. All mice encoded only the wild-type *HPRT* alleles (Thompson *et al.*, 1989). The *Prnp* coding region from 101LL mice was sequenced. The entire ORF was generated by PCR, cloned into a pGEM-T vector (Promega), and plasmid DNA was isolated and sequenced (Sequenase, Amersham).

PCR genotyping of mouse tail DNA

A 765 bp fragment containing the entire *Prnp* ORF was generated using a 5' primer (5'-ATGGCGAACCTTGGCTACTGGCTG-3'); position 107–130, DDBJ/EMBL/GenBank accession No. M18070) and a 3' primer (5'-TCATCCCACGATCAGGAAGATGAG-3'; position 871–848, DDBJ/EMBL/GenBank accession No. M18070). Cycle conditions were: 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C. This was followed by a final 10 min at 72°C (Biometra Triblock). The presence or absence of a *DdeI* site within the PCR product provided a marker for the codon 101P–L alteration.

Southern analysis of PrP gene

Genomic DNA was prepared by lysis of brain tissue (10 mM Tris–HCl pH 7.6, 10 mM EDTA, 0.5% sarcosyl, 10 mM NaCl and 100 µg/ml proteinase K) overnight at 37°C. The lysate was extracted in phenol followed by phenol:chloroform (1:1) and precipitated with isopropanol and resuspended in TE buffer. Following restriction (10 µg of DNA), the DNA fragments were separated on 1% agarose and blotted onto Genescreen Plus (Dupont). Hybridization was carried out at 65°C overnight using a 700 bp *EcoRV*–*Bam*HI fragment (3' probe) and a 884 bp PCR product (Figure 1) as probes. Following stringent washing procedures (0.1 × SSC at 65°C), the blots were exposed to X-ray film for 2 days.

TSE inoculum and challenge

Brain material was obtained from a GSS patient. Genotyping had confirmed the presence of the 102L mutation in one of the alleles of the PrP gene of this patient. The brain material was homogenized (1:10 and 1:100) in normal saline prior to inoculation. Brain material from a 101LL mouse terminally infected with GSS and from the murine scrapie strain of scrapie ME7 were homogenized (1:100) prior to use as inoculum (Fraser and Dickinson, 1968). Mice were coded and genotyped before intracerebral injection with 20 µl of inoculum under halothane anaesthesia. Scoring for signs of illness was performed as described (Dickinson *et al.*, 1968). Mice were genotyped again at post-mortem. All analysis of the mice was carried out prior to decoding the experiment. Incubation periods were calculated as the interval between inoculation and terminal illness.

Lesion profiles

Mice were killed by cervical dislocation at the terminal stage of disease. The brains were removed and fixed in 10% formal saline. H&E-stained coronal sections (6 µm) at four levels were scored for vacuolar degeneration on a scale of 0–5 in nine standard grey matter areas and three standard white matter areas as described (Fraser and Dickinson, 1967, 1968).

PrP immunocytochemistry

Mice were killed by cervical dislocation at the terminal stage of disease. Brains were removed and immersion-fixed for 48 h in formal saline (10%). Fixation was followed by decontamination for 1 h in formic acid (98%). The brains were then dehydrated with alcohol and impregnated in wax during a 7 h processing cycle. Microtome sections (6 µm) were cut and mounted on Superfrost plus slides. Sections were immunostained by the standard peroxidase–anti-peroxidase (PAP) method using two polyclonal antisera to PrP: 1B3 (Farquhar *et al.*, 1989) (1:1000 overnight at room temperature) and 1A8 (Farquhar *et al.*, 1993) (1:400 2 h at room temperature). The reaction product was visualized with diaminobenzidine and sections were counterstained lightly with haematoxylin.

Western blotting

For the detection of PrP^{Sc} in transgenic and wild-type mice, 10% (w/v) homogenates of brain tissue were prepared in NP-40 buffer [0.5% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl pH 7.5]. Homogenates were centrifuged at 11 000 g for 15 min at 10°C to remove cellular debris. Supernatant samples (100 µl) were incubated with or without proteinase K (20 µg/ml) for 1 h at 37°C, and the reaction terminated by the addition of phenylmethylsulfonyl fluoride

(PMSF). Samples were incubated at 90°C for 30 min in SDS–PAGE sample buffer, and separated on 10–15% gradient acrylamide gels using the Neville buffer system (Neville, 1971). Proteins were transferred onto PVDF membrane by electroblotting, and incubated with either monoclonal antibody 8H4 (Zanusso *et al.*, 1998) or polyclonal rabbit serum 1A8 (Farquhar *et al.*, 1993) for 2 h at room temperature. Proteins were visualized with either horseradish peroxidase (HRP)-conjugated rabbit anti-mouse or HRP-conjugated goat anti-rabbit, and a chemiluminescence detection kit (Roche Diagnostics). Membranes were exposed to X-ray film for periods ranging from 10 s to 10 min.

Northern analysis of mRNA from the Prnp^{101L} allele

Total RNA was isolated using RNazol™ B (Biogenesis, Poole, UK) based on the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). A 20 µg aliquot of total RNA was separated on a 1.0% agarose–formaldehyde denaturing gel, transferred to Hybond N (Amersham Pharmacia Biotech, UK) and probed with a ³²P-labelled 936 bp *KpnI*–*EcoRI* fragment from exon 3 of *Prnp*.

Acknowledgements

The authors would like to thank V.Thomson, D.Drummond, K.Wales and J.Beaton for care and scoring of the animals, A.Suttie for tissue processing, A.Chree for lesion profile data, L.Aitchison for technical assistance, G.Miele for RNA analysis, M.Bruce for comments on the manuscript and B.Easter for photography. Antibodies were kindly supplied by C.Farquhar (1B3 and 1A8), M.-S.Sy and P.Gambetti (8H4). This work was supported by a Grant (BS204512) from the Bovine Spongiform Encephalopathy Programme of the BBSRC to D.W.M., J.H. and J.C.M. and an MRC Programme Grant (G9721848) to J.C.M., J.H., M.B. and C.B.

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Received July 20, 1999; revised and accepted October 8, 1999